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TITLE OF THE INVENTION (280 characters max)

CATALYTIC DNA AND ITS USE FOR DETERMINATION OF ANALYTES

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CATALYTIC DNA AND ITS USE FOR DETERMINATION OF ANALYTES

FIELD OF THE INVENTION

This invention relates to a method for detecting an analyte in an assayed sample. More specifically, the present invention concerns catalytic DNA and its use in the determination of an analyte in a liquid medium.

BACKGROUND OF THE INVENTION

The following documents are regarded relevant to the background of the invention:

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The discovery of catalytic RNAs (ribozymes) sparked scientific interest directed to the preparation of new biocatalysts.[1,2] Analogous deoxyribozymes (catalytic DNAzymes) were not found in nature, but synthetic efforts demonstrated the successful preparation of numerous catalytic DNAs for a variety of chemical transformations.[3,4]

An interesting example of a catalytic DNA that revealed peroxidase-like activities is a supramolecular complex between hemin and a single-stranded guanine-rich nucleic acid (aptamer).[5] This complex was reported to catalyze the oxidation of 2,2'-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid, ABTS, by H_2O_2 (a common reaction used for the assay of peroxidase activity). It was suggested[(18)] that the supramolecular docking of the guanine-quadruplex layers facilitates the intercalation of hemin into the complex, and the formation of the biocatalytically active hemin center.

Enzymes[(19)] and, specifically, horseradish peroxidase (HRP)[(20),(21)] are used as biocatalytic labels for the amplified detection of DNA sensing events. The electrochemical amplified detection of DNA was accomplished in the

presence of different enzymes,[(19)(20)] and the chemiluminescence analysis of DNA was reported in the presence of HRP.[(21)]

Nucleic acids in beacon configurations are extensively used as specific DNA sensing matrices. The specific linkage of photoactive chromophores/ quenchers to the hairpin termini results in the chromophore luminescence quenching. The subsequent lighting-up of the chromophore luminescence by the hybridization of the analyzed DNA hairpins and the beacons opening, was used as a general motif for the photonic detection of DNA.[8] The quenching of dyes by molecular or nanoparticle quenchers[9] or the fluorescence resonance energy transfer (FRET) between dyes was used for the optical detection of the hybridization process of the DNA to the beacon. Recently, the labeling of the beacon termini with redox-active units led to the electrochemical detection of hybridization to the hairpins and their ring opening.[10] In that system, ferrocene units were linked to the end of the beacon assembled on an Au electrode. While in the hairpin structure effective electron-transfer between the ferrocene units and the electrode exists, their opening by hybridization with DNA blocks the electrical communication.

GLOSSARY

The term "***nucleic acid peroxidase***" in the context of the present invention means a catalytic nucleic acid sequence capable under the appropriate assay conditions, of peroxidase activity.

The term "***catalytic nucleic acid complex***" in the context of the present invention means a complex of two or more nucleic acid sequences capable, given the necessary assay conditions, of enzymatic activity. Examples for such enzymatic activity are peroxidase activity and ribozyme activity, including cleavage of RNA or DNA, splicing phosphoesterification , porphyrin metallation, and DNA ligation, and peroxidation .

The term "***nucleic acid peroxidase complex***" in the context of the present invention means a complex of one or more nucleic acid sequences capable of

forming a complex that, uner the appropriate assay conditions, is capable of peroxidase activity.

The term "***nucleic acid***" or "***nucleotide***" in the context of this invention means a nucleic acid sequence comprising one or more of dNTPs, rNTPs and/or synthetic analogues thereof.

The term "***determination***" in the context of the present invention means qualitative and/or quantitative detection.

The term "***binding conditions***" in the context of the present invention means any conditions that allow two or more members of a complex forming group to bind together. Such conditions may comprise concentration and/or type of solutes, pH, temperature, presence of additional members of said complex forming group, etc. The term binding conditions may also mean several different conditions applied one after the other allowing a a number of complexes to form and/or dissipate, as necessary.

The term "***assay conditions***" in the context of the present invention means any conditions (or sequences of conditions) that allow desired binding and catalytic activity (or a sequence of binding or catalytic activities) to take place and the desired signal to be formed. The assay conditions include providing a substrate. The substrate is any substrate of the catalyzed reaction that under assay conditions the yields a reaction signal. The assay conditions may also comprise concentration or type of solutes, pH, and temperature. It is noted that in some cases care should be taken that the assay conditions should be such that would not disrupt any binding that was achieved earlier under binding conditions.

In case of nucleic acid peroxidase for example, the assay conditions should allow catalysis of a peroxidase reaction yielding light, color or a sediment, as the case may be. In such case H_2O_2 and, if necessary, a hemin moiety should be provided. It is noted that H_2O_2 may be produced directly or by providing the conditions that allow its formation in the assay solution (e.g. by doxorubicin). The substrate may be for example 2,2'-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) or luminol. In case

of light detection for a peroxidase reaction, the substrate may be any substrate of the nucleic acid peroxidase that would yield light as a result of the peroxidase reaction (e.g. luminol).

The term "***complex forming group***" in the context of the present invention means any group comprising two or more members having a specific affinity towards one another and thus capable of binding together (directly or indirectly) in a selective manner to form a complex. Such groups may comprise for example any of the following: an antigen and an antibody (or an antigen binding portion of an antibody), two complementary nucleic acids, a nucleic acid sequence and its binding protein, an enzyme and its substrate or inhibitor or co-enzyme, components of any known complex (e.g. components of a complex enzyme or protein complexes such as biotin-avidin), a ligand and a receptor or a ligand binding portion of a receptor, a receptor and its inhibitor, a glycoprotein and a lectin, etc. The complex forming group may comprise a plurality of binding components of different types such as a nucleic acid, its complementary nucleic acid, a protein capable of binding the formed double strand, an antibody to said protein, etc. In the context of the present invention, it may be said that the antibody and the first nucleotide sequence are part of a complex forming group and that they bind together indirectly.

The term "***binding***" in the context of the present invention means any type of binding in particular selective binding between molecules, including without limitation, ionic interaction, hydrogen bonds, Van der Waals interaction, covalent binding, etc.

SUMMARY OF THE INVENTION

The present invention is based on the finding that DNAzyme, previously known as a peroxidase where the substrate is ABTS, can also utilize luminol as a substrate thus producing a light emitting product. This finding paves the way to the development of a very sensitive analytic assay wherein the light emission catalyzed by the DNAzyme cam be utilized to detect the presence of an analyte

in a sample. Detection of light is known to be extremely sensitive and thus in accordance with the invention even minute amounts of analytes may be detected.

The method of the invention, as it is bases on a catalytic reaction which amplifies the original signal is therefore very useful for detection of analytes, especially biological molecules in a sample.

The present invention provides a method for determination of an analyte in a sample, said method comprising:

(a) providing a nucleic acid peroxidase;

(b) contacting said nucleic acid peroxidase with said assay sample;

(c) providing assay conditions such that said nucleic acid peroxidase may produce a detectable label dependent on the presence or absence of the analyte;

(d) detecting said light, thereby detecting the presence of the analyte in the assay sample

wherein the method is characterized in that the detectable label produces a light emitting reaction.

Preferably the method is characterized in that that the assay conditions comprise (also) the provision of luminal which produces in the presence of the nucleic acid a light emitting reaction .According to another aspect of the present invention a method is provided for the detection in an assay sample of an analyte, said analyte being one member of a complex forming group, , said method comprising:

(a) providing a nucleic acid peroxidase attached to another member of said complex forming group;

(b) contacting said nucleic acid peroxidase with said assay sample under binding conditions;

(c) removing from the assay sample any nucleic acid peroxidase that is not bound to the analyte;

(d) providing assay conditions such that in the presence of the analyte light is emitted;

(e) detecting said light, thereby detecting the presence of the analyte in the assay sample

The determination according to the present invention can be qualitative detection or quantitative measurement. In both cases care must be taken that the any background luminescence be disregarded. It is therefore preferable that the method contain a step wherein un-bound hemin and/or nucleic acid peroxidase would be removed from the system.

In quantitative detection, the method should also comprise a calibration step in which calibration assay samples containing known concentrations of the analyte are used in order to correlate the light emission and analyte concentration. Such calibration stages are well known in the art.

Thus, in yet another aspect of the invention, a method is provided for the quantification in an assay sample of an analyte, comprising one member of a complex forming group, using a nucleic acid peroxidase, said method comprising:

- providing a nucleic acid peroxidase attached to another member of said complex forming group;
- preparing a calibration scale, said preparation comprising:

  (a) providing at least two calibration assay samples containing known and varying concentrations of the analyte;

  (b) contacting said nucleic acid peroxidase with each of said calibration assay samples under binding conditions;

  (c) removing from the calibration assay sample any nucleic acid peroxidase that is not bound to the analyte;

(d) providing assay conditions such that in the presence of the analyte light is emitted;

(e) detecting light from each calibration assay sample;

(f) deducing a calibration scale;

- detecting presence of the analyte in the assay sample, said detection comprising:

  (a') providing an assay sample;

  (b') contacting the nucleic acid peroxidase with the assay sample under binding conditions;

  (c') removing from the assay sample any nucleic acid peroxidase that is not bound to the analyte;

  (d') providing assay conditions such that in the presence of the analyte light is emitted;

  (e') detecting said light, thereby detecting the presence of the analyte in the assay sample

- comparing the light detected in the assay sample with the calibration scale, thereby quantifying the amount of analyte in the assay sample.

In a preferred aspect of the present invention, the complex comprising the analyte and the nucleic acid peroxidase may be bound to a solid surface such as a plate, slide, chip, bead or bead like structure, magnet, etc. This may, for example, facilitate removal of the unbound nucleic acid peroxidase, as known in the art. The presence or amount of bound peroxidase thus correlates to the presence or amount, respectively of the analyte in the sample

The detection of the present invention as described above and as exemplified below is sufficiently sensitive to detect the difference between two nucleotide analytes having only one (or more) base pair mismatch between them.

Accordingly another aspect of the invention provides a method for the detection in an assay sample of an analyte, being one member of a complex forming group , , said method comprising:

(a) providing conditions for immobilizing the analyte on a solid surface;

(b) providing a nucleic acid peroxidase comprising another member of said complex forming group;

(c) contacting the nucleic acid peroxidase with the solide surface under binding conditions;

(d) removing unbound nucleic acid peroxidase;

(e) providing assay conditions such that in the presence of the analyte light is emitted;

(f) detecting said light, thereby detecting the presence of the analyte in the assay sample.

The analyte may be immobilized to the solid surface by specific or non specific interactions such as by the use of immobilized antibodies, immobilized sequences complementary to part of the analyte.

In the present invention as described in the methods detailed above, the light emitted in accordance with the presence invention may be enhanced or amplified by providing a plurality of nucleic acid peroxidases (at least one of which comprising a member of the complex forming group) being bound to a bead like particle (e.g. gold). Thus, when the nucleic acid peroxidases ,being a member of the complex forming group, is bound to an analyte, the other nucleic acid peroxidases are also bound thereto via the bead like particle and are not removed from the sample along with un-bound nucleic acid peroxidase. Thus all the nucleic acid peroxidases present on the bead-like particle may contribute to the production of the light emitting signal. Accordingly the detection methods of the present invention have two steps of signal amplification one in that each nucleic acid peroxidase can catalyze a plurality of light emitting products and

one that several such nucleic acid peroxidases become bound for each analyte molecule.

In yet another aspect of the present invention, a method is provided for detection of an analyte being one member of a complex forming group in an assay sample, , said method comprising:

(a) providing a pre-catalytic complex comprising a nucleic acid peroxidase sequence attached to an inhibitory moiety being another member of the complex forming group , said inhibitory moiety , in the absence of the analyte sterically hindering the catalytic activity of the catalytic nucleic acid while in the pre-catalytic complex, and said steric hindrance being removed upon binding of the inhibitory moiety to the analyte

(a) contacting said pre catalytic with said assay sample under binding conditions;

(b) providing assay conditions such that in the presence of the analyte light is emitted;

(c) detecting said light, thereby detecting the presence of the analyte in the assay sample.

In a preferred embodiment of the his aspect of the invention, the analyte is a nucleic acid analyte, and the complex forming groups comprise nucleotide sequences being complementary one to the other.

In this aspect the inhibitory moiety is a nucleic acid sequence that in the absence of the analyte sterically hinders (for example due to hybridization) the nucleic acid peroxidase. Only where the analyte is present the inhibitory sequences hybridized therewith thus enabling the nucleic acid peroxidase to exert its catalytic activity.

In a further aspect of the present invention, a method is provided for detection in an assay sample of an analyte being one member of a complex forming group, the method comprising:

(a) providing two or more nucleic acid sequences forming a catalytic nucleic acid complex having in the absence of an analyte ,catalytic activity

which produces a detectable product, at least one of said two or more nucleic acid sequences attached another member of the complex forming group, such that binding of the analyte to the other member attached to the at least one of said two or more nucleic acids prevents the catalytic activity,

(a) contacting said two or more nucleic acids with said assay sample under binding conditions;

(b) providing assay conditions such that in the absence of the analyte the detectable product is produced ;

(c) detecting reaction signal and comparing the reaction signal to a control signal produced in the absence of the analyte ,

decrease of said reaction signal indicating the presence of the analyte in the assay sample.

The determination according to this aspect of the present invention can be qualitative detection or quantitative measurement. In quantitative detection, the method should also comprise a calibration step in which calibration assay samples containing known concentrations of the analyte are used in order to correlate the reduction in reaction signal emission and analyte concentration. Such calibration stages are well known in the art.

Thus, in yet another aspect of the invention, a method is provided for the quantification of an analyte being one member of a complex forming group in an assay sample, using two or more nucleic acids forming a catalytic nucleic acid complex, at least one of said two or more nucleic acids comprising another member of the complex forming group, such that binding of the analyte to the at least one of said two or more nucleic acids prevents the catalytic acitivity, said method comprising:

- providing said two or more nucleic acids;

  preparing a calibration scale, said preparation comprising:

(a) providing at least two calibration assay samples containing known and varying concentrations of the analyte;

(b) contacting said two or more nucleic acids with each of said calibration assay samples under binding conditions;

(c) providing assay conditions such that in the absence of the analyte a reaction signal is emitted;

(d) detecting the reaction signal from each calibration assay sample;

(e) deducing a calibration scale;

- detecting presence of the analyte in the assay sample, said detection comprising:

(a') providing an assay sample;

(b') contacting said two or more nucleic acids with the assay sample under binding conditions;

(c') providing assay conditions such that in the absence of the analyte a reaction signal is emitted;

(d') detecting the reaction signal from the assay sample;

- comparing the reaction signal detected in the assay sample with the calibration scale, thereby quantifying the amount of analyte in the assay sample.

The "*reaction signal*" may be any measurable parameter (or product yielded as a result of the catalyzed reaction. The term "*reaction*" is used to denote one or more reactions or interactions carried out at once or in sequence, to yield the reaction signal. The reaction signal may thus be an electric response including any measurable change in the electrical parameters recorded by or electrical properties

of the electrode. An electric response may be flow of current, charge or potential change, that results from the reaction occurring at the surface of the electrode; a change in the amperometric response of the electrode that can be measured, for example, by means of a cyclical voltamogram; etc. In addition to an electric response, other examples for the reaction signal are the emission of light, a colorimetric response or the formation of a precipitate on a sensing member.

The invention is not limited by the manner in which the reaction signal is measured and any manner of measurement that may be used therefore could be applied for measurement of the electric response in the method of the invention.

In one aspect, each of the two or more nucleic acids forming a catalytic nucleic acid complex also comprises a member of a complex forming group whilst the analyte comprises the other member. In such case, under binding conditions, the analyte may bind (simultaneously or separately) the two or more nucleic acids.

According to a preferred embodiment of the above method, the catalytic nucleic acid complex is a nucleotide peroxidase, formed of at least two nucleic acid fragments of a single strand nucleic acid molecule forming the nucleotide peroxidase. The two or more nucleic acids may form together the whole sequence of the nucleotide peroxidase (e.g. obtainable by splitting the nucleotide peroxidase in two) or at least an active part thereof (e.g. if some bases are removed).

Thus according to a preferred embodiment the present invention provides a method for detection of a nucleic acid analyte being one member of a complex forming group in an assay sample, , said method comprising:

(a) providing said two or more nucleic acid sequences forming together a nucleic acid peroxidase, at least one of said two or more nucleic acids comprising another member of the complex forming group, such that binding of the analyte to the at least one of said two or more nucleic acid sequences prevents the peroxidase activity ;

(b) contacting said two or more nucleic acids with said assay sample under binding conditions;

(c) providing assay conditions such that in the absence of the analyte a reaction signal is emitted;

(d) detecting said reaction signal, thereby detecting the presence of the analyte in the assay sample.

The reaction signal in this case may be for example any measurable parameter resulting, directly or indirectly, form the peroxidase reaction. Examples for such signals are a colorimetric product (wherein the substrate is ABTS) or light (wherein the substrate is luminol).

In a one aspect of the present invention, the nucleic acid complex may be bound to a solid surface such as a plate, slide, chip, bead or bead like structure, magnet, etc. This may, for example, allow measurement of the nucleic acid complex' activity via measurement of a precipitate resulting from the catalyzed reaction, as known in the art.

Accordingly another aspect of the invention provides a method for detection in an assay sample of an analyte being one member of a first complex forming group, , said method comprising:

(a) providing said two or more nucleic acid sequences forming a catalytic nucleic acid complex, at least one of said two or more nucleic acids comprising another member of said first complex forming group, and at least one of said two or more nucleic acids comprising a member of a second complex forming group, such that binding of the analyte to the at least one of said two or more nucleic acids prevents the catalytic activity;

(b) providing a solid surface having bound thereto a member of said second complex forming group and contacting said solid surface with said two or more nucleic acids under

binding conditions such that the nucleotide peroxidase becomes bound to the solid surface;

(c) contacting said solid surface with the assayed sample under binding conditions such that the analyte binds to the at least one of said two or more nucleic acids that comprises the other member of the first complex forming group;

(d) providing assay conditions such that in the absence of the analyte a reaction signal is emitted;

(e) detecting said reaction signal, thereby detecting the presence of the analyte in the assay sample.

DESCRIPTION OF THE INVENTION

Recently we demonstrated that the hemin/G-quadruplex mimics peroxidase by the generation of chemiluminescence,[7] and this property was used for the development of labels for DNA detection. The use of DNAzymes as catalytic labels for biosensing is particularly attractive since non-specific adsorption processes, often encountered with protein-based labels, may be eliminated.

The integration of a DNA biocatalyst into DNA detection schemes could provide a new method for the detection of nucleic acids that might reveal several important advantages: (i) The catalytic DNA may substitute the protein-based biocatalysts, and thus eliminate non-specific binding phenomena. (ii) Tailoring of the DNA biocatalyst as a part of the labeled nucleic acid may reduce the number of analytical steps for detection of the DNA. Here we report that two separated nucleic acids that include the segments A and B, that constitute the single stranded peroxidase deoxyribozyme that forms a layered G-quadruplex structure[(22)], self-assemble in the presence of hemin to form a biocatalyst for the generation of chemiluminescence in the presence of H_2O_2 and luminol. We study the effect of hybridization on the resulting biochemiluminescence.

We demonstrate the self-assembly of biocatalytic hemin/nucleic acid supramolecular complexes on Au-electrodes in monolayer configurations, and describe the biocatalytic and bioelectrocatalytic formation of chemiluminescence at the interfaces. We also describe the inhibition or blocking of the light emission processes by hybridization.

The development of catalytic beacons may provide a major advance in DNA sensing. Here we wish to report on the tailoring of catalytic beacons for the sensing of DNA and telomerase activity originating from HeLa cancer cells. We design beacon structures that upon opening the hairpin by hybridization yield in the presence of hemin a DNAzyme that allows the biocatalytic analysis of the hybridization process.

In conclusion, the present invention has introduced a new concept of catalytic beacons for analyzing DNA and telomerase activity. Besides the importance of the catalytic beacons as a bioanalytical tool, the functional beacons used for the assay of telomerase activity reveal a logic processable function. The beacon opening and the activation of its catalytic properties will occur only provided that the telomers are processed. Thus, the activated catalytic functions of the DNAzyme mimic an "AND" logic gate, where the biocatalytic properties are observed if the telomers are processed and the beacon is opened. Thus, the present system complements recent activities directed to the use of DNA reactions as analogs for logic gates.[18] We also design beacon structures that upon opening the hairpin by hybridization yield in the presence of hemin a DNAzyme that allows the biocatalytic analysis of the hybridization process.

DESCRIPTION OF THE FIGURES

Figure 1A. Analysis of DNA by opening of a beacon nucleic acid and the generation of a DNAzyme.

Figure 1B. Analyzing telomerase activity by a functional DNA beacon that self-generates a DNAzyme.

Figure 2 Absorbance changes originating from the formation of **(4)** upon analysis of: (a) **(2)**, 4.3 μM. (b) color generated by hemin and **(2)**, 4.3 μM, in the absence of **(1)**. (c) Color formed by hemin and **(1)** without **(2)**. (d)-(f) Analysis of variable concentrations of **(2)** corresponding to 3.0 μM, 2.15 μM, 1.30 μM, 0.40 μM and 0.2 μM, respectively. (i) and (j) The analysis of the SPM mutations **(2a)** or **(2b)**, 4.3 μM. All experiments were performed in the presence of **(1)**, 0.43 μM, hemin, 0.43 μM, ABTS, 3.2 mM and H_2O_2, 3.2 mM in a 0.1 M tris buffer solution, pH=8.1 that included $MgCl_2$ 20 mM. Inset: Calibration curve corresponding to absorbance of the system upon analyzing variable concentrations of **(2)** after a fixed time-interval of 60 seconds.

Figure 3 Absorbance changes upon analyzing telomerase activity originating from: (a) 10,000 HeLa cells, (b) 10,000 heat-treated HeLa cells (95°C, 10 minutes). In all experiments the systems consisted of the catalytic beacon **(5)**, 0.04 μM, hemin, 0.04 μM, ABTS, 3.2 mM and H_2O_2, 3.2 mM in 0.1 tris buffer solution, pH=8.1 that included $MgCl_2$, 20 mM. Inset: Calibration curve corresponding to absorbance change of the system upon analyzing variable numbers of HeLa cells.

Figure 4 Chemiluminescence generated by a nucleic acid/hemin supramolecular complex and the inhibition of the DNAzyme activity by hybridization.

Figure 5 The reconstitution of nucleic acids on a hemin monolayer modified surface and the generation of a biochemiluminescence DNAzyme and the inhibition of the process by hybridization.

Figure 6 The assembly of a nucleic acids/hemin complex on an electrode for the electrochemical generation of chemiluminescence and the inhibition of the process by hybridization.

Figure 7 Integrated photons recorded in the systems consisting of: (a) The nucleic acids (**1**) and (**2**) each 12.5 μM, and hemin 12 μM. (b) Hemin 12 μM, (c) The nucleic acids (**1**) and (**4**) 12.5 μM, and hemin, 12 μM. (d) (**4**) 12.5 μM, without hemin. (e) to (h) (**1**) and (**2**) 12.5 μM each, hemin 12 μM and competitive hybridizing nucleic acid (**4**) at concentrations that correspond to 3 μM, 6 μM, 9.5 μM and 12.5 μM, respectively. (i) (**4**) 12.5 μM and hemin 12 μM without added (**1**) and (**2**).

Figure 8 Integrated photons emitted by (a) The hemin-modified surface reconstituted with (**1**) and (**2**), 2.5 μM each. (b) The hemin-modified surface without the reconstitution with (**1**) and (**2**). (c) The hemin-modified surface reconstituted with (**1**) or (**2**), 2.5 μM each. (d) to (g) The hemin-modified surface reconstituted with (**1**) and (**2**), 2.5 μM each in the presence of (**4**) at concentrations corresponding to 0.6 μM, 1.2 μM, 1.9 μM, 2.5 μM, respectively.

Figure 9 Time-dependent photons counted in the system consisting of: (a) The (**6**)/(**7**)-functionalized electrode treated with hemin, 1.2 μM, and doxorubicin, (**5**), 5 μM. (b) The (**6**)-functionalized electrode. (c) The (**6**)-functionalized electrode hybridized with (**8**), 2.5 μM. In experiments (b) to (d) the resulting electrodes were treated with hemin, 1.2 μM, and doxorubicin, 5 μM. Inset: The calibration curve that corresponds to the percent of photons as a function of the different concentrations of (**8**) corresponding to 0μM, 1.2μM, 1.9μM, 2.5μM, respectively. Light emission was

detected upon the application of the potential corresponding to -0.6 V vs. SCE.

Figure 10 depicts the analysis of DNA by opening of a beacon nucleic acid and the generation of a DNAzyme

Figure 11 Absorbance changes originating from the formation of (4) upon analysis of: (a) (2), 4.3 μM. (b) color generated by hemin and (2), 4.3 μM, in the absence of (1). (c) Color formed by hemin and (1) without (2). (d)-(f) Analysis of variable concentrations of (2) corresponding to 3.0 μM, 2.15 μM, 1.30 μM, 0.40 μM and 0.2 μM, respectively. (i) and (j) The analysis of the SPM mutations **(2a)** or **(2b)**, 4.3 μM. All experiments were performed in the presence of (1), 0.43 μM, hemin, 0.43 μM, ABTS, 3.2 mM and H_2O_2, 3.2 mM in a 0.1 M tris buffer solution, pH=8.1 that included $MgCl_2$ 20 mM. **Figure 11, inset**: Calibration curve corresponding to absorbance of the system upon analyzing variable concentrations of (2) after a fixed time-interval of 60 seconds.

Figure 12 Shows the method of the invention wherein a plurality of nucleic acid peroxidases are present on a "bead-like" gold structure for amplification of the signal.

Figure 13 Shows the light emission reading with varying concentrations of the "bead-like" gold structure described in Fig 12.

EXPERIMENTAL SECTION

Figure 1A depicts the method for the application of the beacon (1) as a catalytic unit for the sensing of DNA (2). The hairpin structure of (1) includes the sequence consisting of segments A and B that in an open configuration forms a G-quadruplex complex with hemin that reveals peroxidase-like activity (structure

I in Figure 1A). Since the segment B is hybridized in the hairpin structure, the formation of the catalytic DNAzyme is prohibited. Hybridization of the analyzed DNA, (2), with the hairpin structure opens the beacon and the released sequence of nucleic acids (components A and B) self-assemble in the presence of hemin to form the catalytic DNA that catalyzes the oxidation of 2,2'-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid, ABTS, (3), to the colored product (4) by H_2O_2. The hybridization and hairpin opening is then detected spectroscopically by following the accumulation of (4) at $\lambda = 414$ nm ($\varepsilon = 36$ mM^{-1} cm^{-1}). Figure 2, curve (a), shows the time-dependent color evolution upon the analysis of the DNA (2) 4.28 μM. The control experiment that follows the spectral changes of the hairpin (1) in the presence of hemin, H_2O_2 and ABTS, does not lead to any development of a color due to the oxidation of (3). Also, the hybridization of (2) with a hairpin structure that lacks the B segment in the "hairpin stem" does not lead to an active DNAzyme. These results clearly indicate that only upon the hybridization of (2) with the beacon (1) and its opening, the DNAzyme that stimulates the oxidation of ABTS is generated. The extent of opening of the sensing beacons, and thus, the quantity of the generated DNAzyme, is controlled by the concentration of (2). Figure 2, curve (c) to (h) show the time-dependent evolution of the oxidized product (4), at variable concentrations of the analyzed DNA. As the concentration of (2) increases, the formation of (4) is enhanced.

Figure 2, inset, shows the extracted calibration curve that shows the color developed by the system upon analyzing variable concentrations of (2) and monitoring the color accumulated by the biocatalyzed oxidation of (3) after a fixed time-interval of 60 seconds. As expected, the biocatalytic process is enhanced as the concentration of (2) increases. The catalytic beacon reveals specificity and single base mismatches may be discerned. For example, Figure 2, curves (i) and (j) show the time-dependent accumulation of (4) upon analyzing the mutants (2a) and (2b), 4.28 μM that include a single-base mismatch relative to the fully complementary analyzed DNA, (2). Clearly, the signal for analyzing

(2) is 8-fold higher than the signal for the mutants (after 4 minutes of product accumulation).

Telomers are nucleic acids consisting of constant repeat units at the ends of human chromosomes.[11] The telomers protect the chromosomes, and their sequential erosion during cell proliceration provides a cellular signal for the termination of the cell life cycle. Telomerase is a ribonucleoprotein that includes an RNA component that acts as a template for the replication of the 3'-ends of the linear chromosomes with the telomere repeat units.[12] The accumulation of telomerase in cells results in the constant addition of the telomere repeats, turning them into immortal cells of uncontrolled growth. Indeed, in most cancer and malignant cells increased levels of telomerase were detected, and telomerase is considered as an important biomarker for cancer and malignant cells.[13] Several analytical procedures for the determination of telomerase activity have been developed, including the telomeric repeat amplification protocol, (TRAP), that involves PCR amplification,[14] the functionalization of the telomers with fluorescent labels,[15] and the electrochemical[16] or surface plasmon resonance (SPR)[17] readout of the telomerization process. We have applied a catalytic beacon as an active component of the analysis of telomerase activity. Figure 1B depicts the method to analyze the telomerase activity by means of a catalytic beacon. The beacon, (5), is designed to include at its two termini two functional nucleic acid components. One end of the hairpin structure is ends with a nucleic acid that includes the base sequence that is a part of the DNAzyme in the presence of hemin (part A). The second part of the DNAzyme base sequence (part B) is "hidden" in the hybridized hairpin configuration. At the other end of the hairpin, a nucleic acid segment that is a primer (6) for telomerase, and for the initiation of the telomerization, is tethered to the beacon (part C of the beacon). The single stranded loop of the beacon is complementary to the telomere repeat units. Treatment of the beacons with HeLa cancer cell extract in the presence of the dNTP nucleotide mixture, results in the telomerization of the hairpin end. The elongated telomere self-generates the sequence for its hybridization with the

complementary hairpin loop, and leads to the beacon opening, and to the generation of the DNAzyme. Thus, the telomerase activity is monitored by following the ABTS oxidation by H_2O_2 upon the hairpin structure opening.Figure 3, curve (a), shows the time-dependent accumulation of the colored product (4) upon analyzing telomerase originating from 10,000 cells. Figure 3, curve (b), shows the results of the control experiment where the accumulation of (4) from a system that included heat-treated (95°C for 10 minutes) of 10,000 HeLa cell extract (the telomerase in the cells is deactivated upon heating). Clearly, the DNAzyme is not formed, and no color of (4) is developed in the system. The rate of the telomeres' formation is controlled by the content of telomerase in the sample, and thus the accumulation of (4) is regulated by the number of HeLa cells that are analyzed. Figure 3, insert, shows the absorbance values of (4), obtained upon the analysis of the telomerase activity originating from different numbers of HeLa cells. (The absorbance of generated (4) is determined after a fixed time of telomerization corresponding to 8 minutes).

The two nucleic acids in **(1)** and **(2)** include the segments A and B that could self-assemble on the hemin site. Treatment of hemin (12 μM) with the two nucleic acids **(1)** and **(2)**, 12.5 μM each, results in the formation of a 1:1:1 supramolecular complex, $K_d = 130\ \mu M^2$, Figure 4. This supramolecular complex reveals biocatalytic functions and in the presence of H_2O_2 and luminol, **(3)**, the system generates chemiluminescence. Figure 7, curve (a), shows the integrated light intensity emitted from the system. Control experiments reveal that the formation of the supramolecular complex is essential to generate the light emission. Hemin itself yields very low chemiluminescence, curve (b), and hemin in the presence of the separated nucleic acids **(1)** and **(4)**, generates a very low light output, curve (c). (The hybridization of **(2)** and **(4)** in the presence of hemin leads to negligible light emission). Also, the nucleic acid **(4)** in the absence of hemin does not yield any light emission, curve (d). These results indicate that the self-assembly of **(1)** and **(2)** with hemin is essential to generate the biocatalyzed

light emission. The nucleic acid chains linked to the segments A and B of (1) and (2) are complementary to the 5' and 3' ends of the nucleic acid (4). Figure 7, curves (e) to (h) show the effect of hybridization of (4) with the nucleic acids (1) and (2) in the presence of hemin on the emitted light intensity. Evidently, as the concentration of (4) increases, the biocatalytic light emission decreases, and at a concentration of 12.5 μM, 70 % of the original chemiluminescence is blocked. A control experiment that examined the light emission from the nucleic acid (4) in the presence of hemin revealed low level chemiluminescence, Figure 7, curve (i). Thus, the decrease in the chemiluminescence generated by the system consisting of the supramolecular complex hemin/(1)/(2) upon addition of (4) is attributed to the separation of the biocatalytically-active DNAzyme upon hybridization to (4). Presumably, hybridization of (1) and (2) with (4) distorts the segments A and B to a configuration that cannot form the biocatalyst structure.

The biocatalytic generation of chemiluminescence was also examined on the surface. Hemin was covalently linked to a Au-surface, Figure 5. The tethered hemin units were then used as sites for the reconstitution of the biocatalytic peroxidase-like supramolecular complex on the surface, by the interaction of the functionalized surface with nucleic acids (1) and (2). Coulometric assay of the redox-wave of the heme units indicates a surface coverage of 3.5×10^{-11} mole·cm^{-2}. Thus, ca. 18 % of the hemin units are reconstituted with the nucleic acids (1) and (2). Figure 8, curve (a), shows the integrated light intensity emitted by the DNAzyme interface in the presence of H_2O_2 and luminol. Control experiments confirm that very low light emission is stimulated by the hemin monolayer alone, curve (b), and that hemin in the presence of (1) or (2) alone does not lead to any significant chemiluminescence, curve (c). Figure 8, curves (d) to (g), show the light emitted from the system in the presence of different concentrations of added (4). As the concentration of (4) increases, the emitted light intensity decreases. Microgravimetric quartz crystal microbalance experiments indicate that the hybridization of the free nucleic acid parts of (1) and (2) with (4) leads to the dissociation of the hemin-nucleic acids

complex from the surface, and at a concentration of **(4)** that corresponds to 2.5 μM, the crystal frequency is almost similar to the hemin-monolayer-functionalized crystal prior to the reconstitution with **(1)** and **(2)**. Thus, the hybridization of **(1)** and **(2)** with **(4)** presumably distorts the segments A and B leading to the dissociation of the surface-confined biocatalytic supramolecular complex.

A further surface confined biocatalytic system for the generation of chemiluminescence in the presence of the DNAzyme was designed by the *in situ* generation of H_2O_2. Previous studies have demonstrated that the intercalation of doxorubicin, **(5)**, into the double-stranded DNA associated with an electrode, allows the electrocatalyzed reduction of O_2 to H_2O_2 by the intercalated quinone, and the subsequence light emission in the presence of HRP and luminol.[9] Figure 6 shows the assembly of the DNAzyme system on an electrode for the biocatalyzed generation of chemiluminescence, and its application for the analysis of a nucleic acid. The thiolated nucleic acid, **(6)**, that includes the nucleic acid component "A" of the DNAzyme, is assembled on the electrode. The hybridization of the non-enzymatic part of **(6)** with the complementary part of the nucleic acid **(7)**, that includes the segment "B" of the DNAzyme, yields the interface that binds hemin and generates the peroxidase mimicking DNAzyme. The intercalation of doxorubicin, **(5)**, to the double-stranded DNAzyme produces the bioelectrocatalytic interface for the electrocatalyzed light emission. The electrocatalyzed reduction of **(5)** produces H_2O_2 and the DNAzyme catalyzes the emission of light in the presence of luminol. Microgravimetric quartz crystal microbalance experiments indicate that the surface coverage of the thiolated nucleic acid **(6)** is 9.5×10^{-12} mole·cm^{-2}, and of the double-stranded nucleic acid structure **(6)**/**(7)** is 4.6×10^{-12} mole·cm^{-2}. Coulometric assay of doxorubicin **(5)**, response indicated a surface coverage of ca. 2.8×10^{-11} mole·cm^{-2}. Thus ca. six doxorubicin units are intercalated into each double-stranded DNA. Figure 9, curve (a), shows the time-dependent light intensity emitted by the system upon the applying a potential of -0.6 V vs. SCE on the electrode. This potential

reduces the doxorubicin associated with the double-stranded DNA on the surface. Doxorubicin mediates the catalyzed generation of H_2O_2 during the reduction process and the electrogenerated H_2O_2 leads to biochemiluminescence in the presence of luminol, (3). Control experiments indicate that no light emission is observed upon application of the same sequence of reactions on the (6)-modified surface without hybridization with (7) (Figure 9, curve (b)). A further control experiment shows the light emitted from the system consisting of the (6)-functionalized electrode upon interaction with (8) that lacks the segment "B" of the DNAzyme, 2.5 μM, that is further treated with hemin and doxorubicin (5), and subjected to the potential of -0.6 V in the presence of luminol (Figure 9, curve (c)). Clearly, the emitted light intensity is negligible, implying that hybridization of (8) with the interface inhibits the formation of the biocatalytic interface for chemiluminescence. Also, the interaction of the mercaptohexanol-functionalized surface with (8) and then with hemin and doxorubicin followed by the application of the potential of -0.6 V vs. SCE in the presence of luminol, (3), did not yield any electrogenerated chemiluminescence (Figure 9, curve (d)). Thus, the control experiments reveal that the hybridization between (6) and (7) is essential to form the complex with hemin and to intercalate doxorubicin (5), into the double stranded assembly. The electrochemical reduction of the intercalator supplies the H_2O_2 for the DNAzyme and this activates the light emission process. Addition of the nucleic acid (8) that is complementary to the surface-associated nucleic acid (6) competes with (7) towards the hybridization process. Since (8) lacks the "B" part for the self-assembly of the biocatalytic complex with hemin, the light emission in the presence of hybridized (8) should be blocked. Figure 9, inset, shows the calibration curve that corresponds to the light intensities emitted by the (6)-modified electrode upon hybridization with (7) in the presence of different concentrations of (8), and upon treating the interface with hemin and (5) and applying the reductive potential in the presence of luminol, as described above. The advantages of using DNAzymes as catalytic labels for the analysis of DNA rest, in the enhanced specificity of the analytical protocols. While the use

of enzymes and enzyme conjugates always involves non-specific adsorption, the application of nucleic acid catalysts eliminates the phenomenon. One important aspect of the present study is the demonstration that self-assembly of two specific nucleic acids and hemin may yield a supramolecular biocatalytic entity.

In conclusion, the present study has revealed the novel functions of a hemin-nucleic acid supramolecular complex as a DNAzyme that reveals peroxidase-like chemiluminescence activities. Besides the fundamental interest in the DNAzyme activities of the systems, the systems have important practical implications since the chemiluminescence DNAzyme may act as an internal nucleic acid biocatalytic label for DNA sensing. That is, one may design protein-less amplified DNA detection scheme using chemiluminescence as a transduction means.

Materials. Hemin was purchased from Porphyrin Products (Logan, Utah), and used without further purification. A hemin stock solution was prepared in DMSO, and diluted in DMSO. Solutions were frozen and stored in the dark at -20°C. The concentration of hemin solutions was determined using standard spectroscopic methods.[11] 5-Amino-2,3-dihydro-1,4-phthalazinedione (luminol) and other chemicals were obtained from Sigma and used as supplied. All buffers used for analyzing the DNAzyme chemiluminescent activities contained the non-ionic detergent Triton X-100 (0.05%, w/v) and 1% DMSO.

Nucleic acids were synthesized by Sigma Genosys. They were purified using the PAGE method. The sequences of the oligomers are given below:

(1) 5'-CGATTCGGTACTGGCTCAAAATGRGGAGGGT-3'

(2) 5'-AGGGACGGGAAGAAAGATAATGCGCATGCTCAA-3'

(4) 5'-TTGAGCATGCGCATTATCTGAGCCAGTACCGAATCG-3'

(6) 5'-HS$(CH_2)_6$CGATTCGGTACTGGCTCAAAATGRGGAGGGT-3'

(7) 5'-AGGGACGGGAAGATGAGCCAGTACCGAATCG-3'

(8) 5'-TGAGCCAGTACCGAATCG-3'

Preparation of DNA-Hemin Complexes. Nucleic acid (1), 25 μM, nucleic acid (2), 25 μM, and competitive hybridizing nucleic acid (4) at concentrations that correspond to 0 μM, 6 μM, 12 μM, 19 μM and 25 μM, respectively, were heated to 95°C for 9 min in 10 mM Tris-HCl, pH 7.4, to dissociate any intermolecular G-quadruplex, and allowed to cool to room temperature. An equal volume of the hybridization buffer (50 mM HEPES, 40 mM KCl, 400 mM NaCl, 0.1% Triton X-100 and 2% DMSO, pH 7.4) was added to the mixtures of the nucleic acids and the systems were allowed to hybridize and fold overnight at room temperature. Hemin, 12 μM, was then added to the systems (final DMSO % less than 2%) to form the G-quadruplex structures (12 h, room temperature).

Immobilization of Hemin as a Monolayer and the Reconstitution of DNAzyme on the Gold Surface. The Au-coated (50 nm gold layer) glass plate (22 mm × 11 mm) was immersed into a piranha solution (consisting of 70% concentrated sulfuric acid and 30% hydrogen peroxide) for 20 min, and afterwards thoroughly rinsed with triple-distilled water. The plate was then soaked in concentrated nitric acid for 5 min, and rinsed with water again. The plate was treated with an ethanol solution of 3-mercaptopropionic acid, 1×10^{-2} M, that contained 6-mercaptohexanol, 1×10^{-3} M, for 12 h, and afterwards rinsed with ethanol to remove any non-specifically adsorbed material. The covalent coupling of 1,10-diaminodecane to the thiol monolayer-modified plate was performed by soaking the plate in 0.01 M HEPES buffer solution, pH 7.2, that included 1,10-diaminodecane, 5×10^{-4} M, and EDC, 1×10^{-3} M, for 2 h at room temperature. The resulting plate was washed with 0.01 M HEPES buffer (pH 7.2), and incubated in 0.01 M HEPES buffer solution (pH 7.2) that included hemin, 5×10^{-4} M; Triton X-100, 0.05 %; DMSO, 1% and EDC, 1×10^{-3} M, for 4 h at room temperature. The resulting plates were then rinsed with the hybridization buffer. A mixture of nucleic acid (1), 25 μM, and nucleic acid (2), 25 μM, was heated to 95°C for 9 minutes in 0.01 M Tris buffer (pH 7.4), and

allowed to cool to room temperature. An identical volume of the hybridization buffer was added to the nucleic acid mixture to allow proper folding (12 h, room temperature) The hemin-modified electrode was then immersed in the nucleic acid solution, 2.5 μM, for surface reconstitution (12 h, room temperature). The resulting surface reconstituted hemin/nucleotide complex layer was then interacted with (**4**) (0 μM, 0.6 μM, 1.2 μM, 1.9 μM and 2.5 μM) in a 0.1 M phosphate buffer that included 25 mM HEPES, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100 and 1% DMSO, pH 7.4 (12 h, room temperature).

Immobilization of (6) on the Au-surface and Its hybridization with (7) or (8). The Au-plate was reacted with a 0.4 M phosphate buffer solution, pH 7.4, of (**6**), 6 μM, (12 h), and the resulting surface was then treated with a 0.1 M phosphate buffer solution of 1-mercaptohexanol, 1 mM (1 h). The resulting monolayer-functionalized surface was then treated with the complementary nucleic acids (**7**), 2.5 μM, and (**8**), various concentrations that correspond to 0 μM, 1.2 μM, 1.9 μM and 2.5 μM in a solution composed of 0.1 M phosphate buffer and the perfect Hyb™ hybridization buffer (Sigma), 1:1, 5 h, to yield the ds-DNA assembly on the surface. The resulting surfaces were rinsed with the hybridization buffer and immersed in a 1.2 μM hemin buffer solution that included 25 mM HEPES, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100 and 1% DMSO, pH 7.4 (12 h, room temperature). The resulting system was further treated with doxorubicin (**5**), 5 μM in 0.1 M phosphate buffer, pH 7.4 (1 h, room temperature).

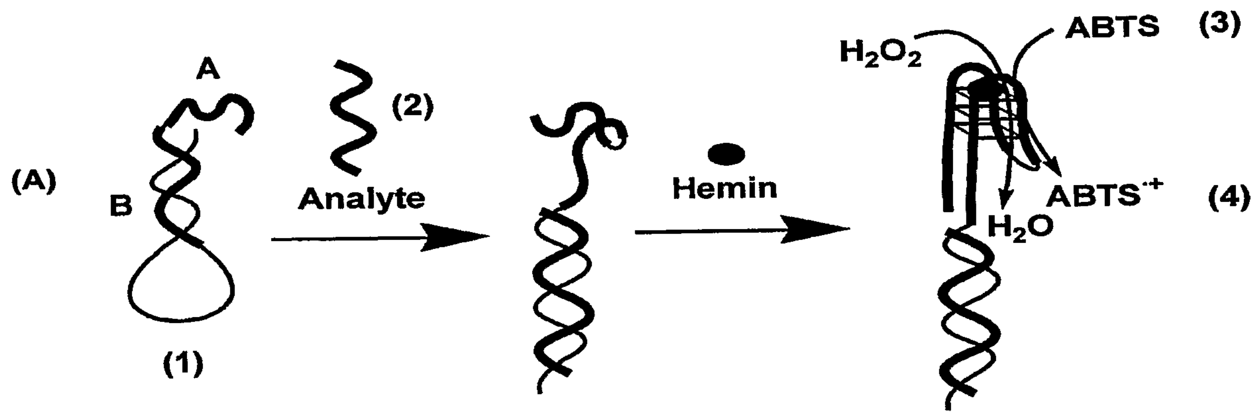
Light Emission Measurements. Light emission was performed by using a photon counting spectrometer (Edinburgh Instruments, FLS 920) equipped with a cooled photomultiplier detection system, connected to a computer (F900 v. 6.3 software). Before the samples analyses the background light was recorded and integrated and this was subtracted from the recorded integrated spectra of the respective samples. Sample analyses were performed by taking 15 μL of the respective DNAzyme solution or the respective modified surfaces into a cuvette that included 3.3 mL of a buffer solution consisting of 25

mM HEPES, 20 mM KCl and 200 mM NaCl, pH = 9.0, that included 0.5 mM luminol and 30 mM H_2O_2.

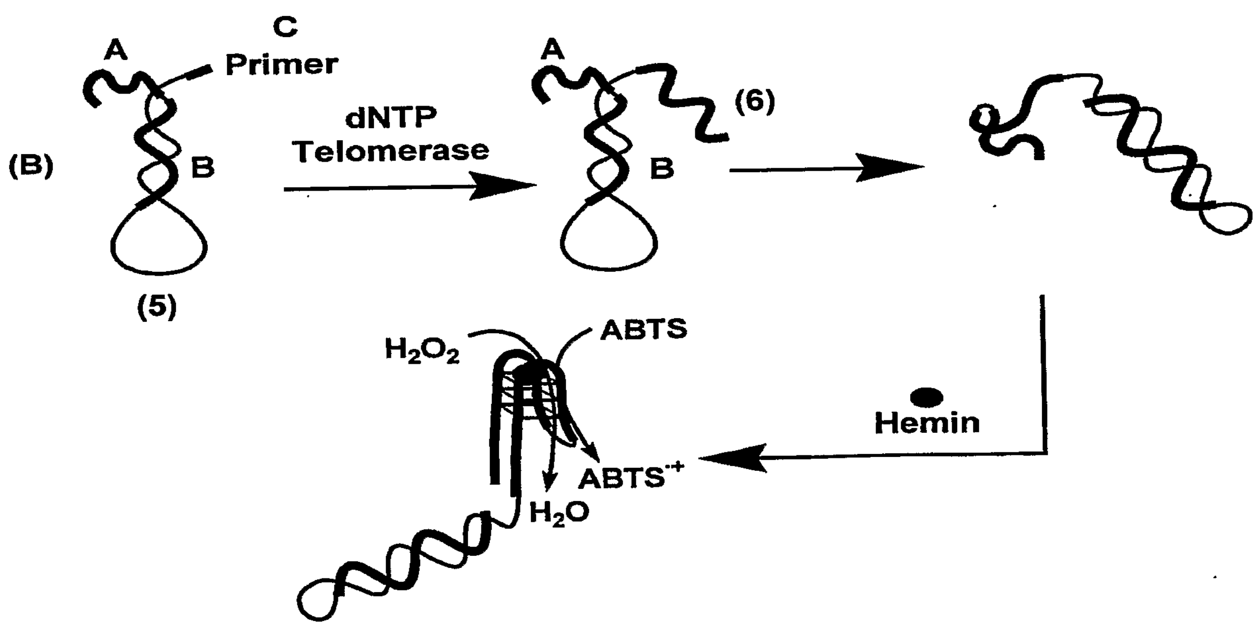
Figure 10 depicts the method for the application of the beacon (1) as a catalytic unit for the sensing of DNA (2). The hairpin structure of (1) includes the sequence consisting of segments A and B that in an open configuration forms a G-quadruplex complex with hemin that reveals peroxidase-like activity (structure I in Figure 10). Since the segment B is hybridized in the hairpin structure, the formation of the catalytic DNAzyme is prohibited. Hybridization of the analyzed DNA, (2), with the hairpin structure opens the beacon and the released sequence of nucleic acids (components A and B) self-assemble in the presence of hemin to form the catalytic DNA that catalyzes the oxidation of 2,2'-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid, ABTS, (3), to the colored product (4) by H_2O_2. The hybridization and hairpin opening is then detected spectroscopically by following the accumulation of (4) at $\lambda = 414$ nm ($\varepsilon = 36$ mM^{-1} cm^{-1}). Figure 11, curve (a), shows the time-dependent color evolution upon the analysis of the DNA (2) 4.28 μM. The control experiment that follows the spectral changes of the hairpin (1) in the presence of hemin, H_2O_2 and ABTS, does not lead to any development of a color due to the oxidation of (3). Also, the hybridization of (2) with a hairpin structure that lacks the B segment in the "hairpin stem" does not lead to an active DNAzyme. These results clearly indicate that only upon the hybridization of (2) with the beacon (1) and its opening, the DNAzyme that stimulates the oxidation of ABTS is generated. The extent of opening of the sensing beacons, and thus, the quantity of the generated DNAzyme, is controlled by the concentration of (2). Figure 11, curve (c) to (h) show the time-dependent evolution of the oxidized product (4), at variable concentrations of the analyzed DNA. As the concentration of (2) increases, the formation of (4) is enhanced.

Figure 11, inset, shows the extracted calibration curve that shows the color developed by the system upon analyzing variable concentrations of (2) and monitoring the color accumulated by the biocatalyzed oxidation of (3) after a

fixed time-interval of 60 seconds. As expected, the biocatalytic process is enhanced as the concentration of **(2)** increases. The catalytic beacon reveals specificity and single base mismatches may be discerned. For example, Figure 11, curves (i) and (j) show the time-dependent accumulation of **(4)** upon analyzing the mutants **(2a)** and **(2b)**, 4.28 μM that include a single-base mismatch relative to the fully complementary analyzed DNA, **(2)**. Clearly, the signal for analyzing **(2)** is 8-fold higher than the signal for the mutants (after 4 minutes of product accumulation).



(1)=5'-CCCTACCCAGCCTTAACTGTAGTACTGGTGAAATTGCTGCC ATTTGGGTAGGGCGGGTTGGG-3'
(2) = 5'-AATGGCAGCAATTTCACCAGTACTACAGTTAAGGC-3'
(2a) = 5'-AATCGCAGCAATTTCACCAGTACTACAGTTAAGGC-3'
(2b) = 5'-AATGGCAGCAATTTCAC GAGTACTACAGTTAAGGC-3'



(5)=5'-TGGGTAGGGCGGGTTGGGAAATAACCCTAACCCTAACCCT AACCCTAACCCTAACCCAACCCAATCCGTCGAGCAGAGTT -3'
(6) = 5'-AATCCGTCGAGCAGAGTTAG(GGTTAG)n-3'

FIGURE 1
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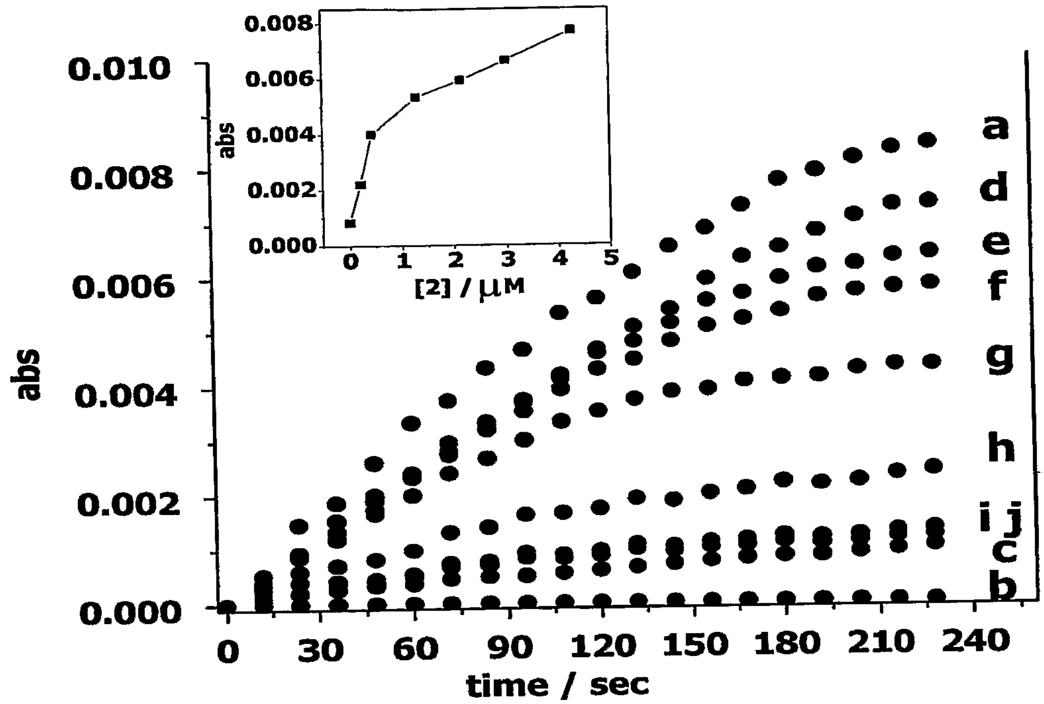


Figure 2

01512474\13-01

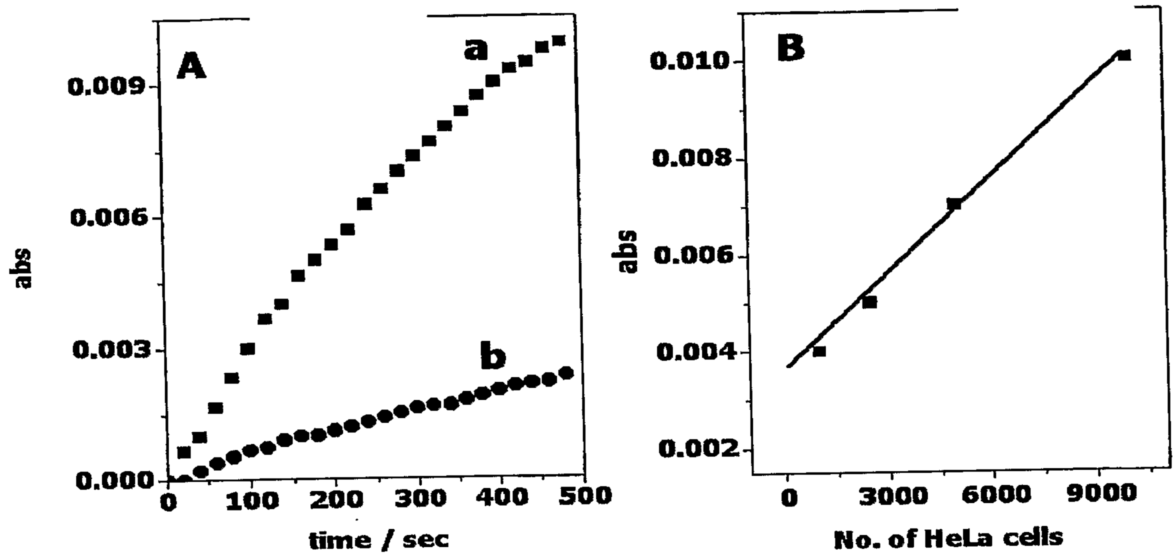
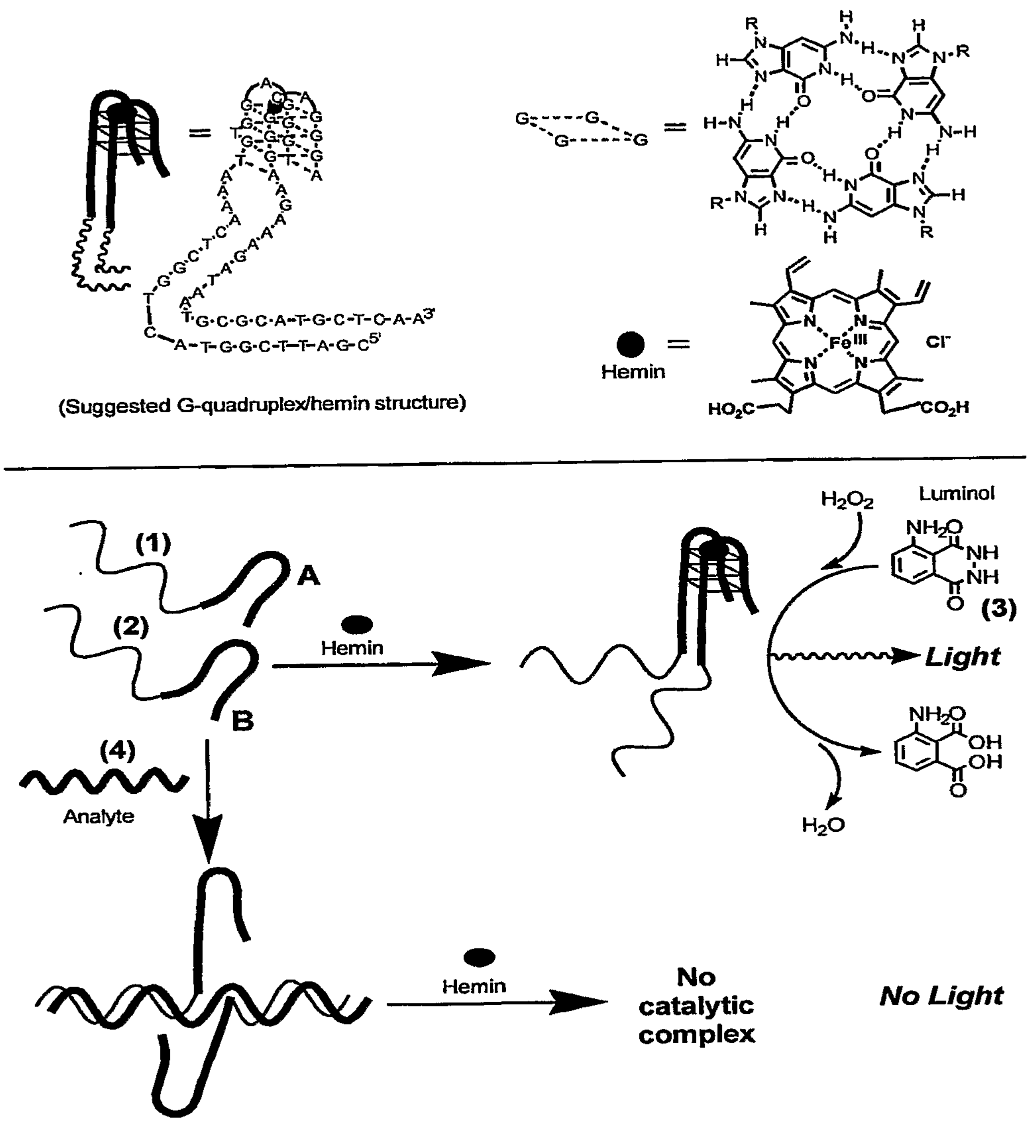

A
a
b
0.009
0.006
0.003
0.000
abs
0 100 200 300 400 500
time / sec
B
0.010
0.008
0.006
0.004
0.002
abs
0 3000 6000 9000
No. of HeLa cells

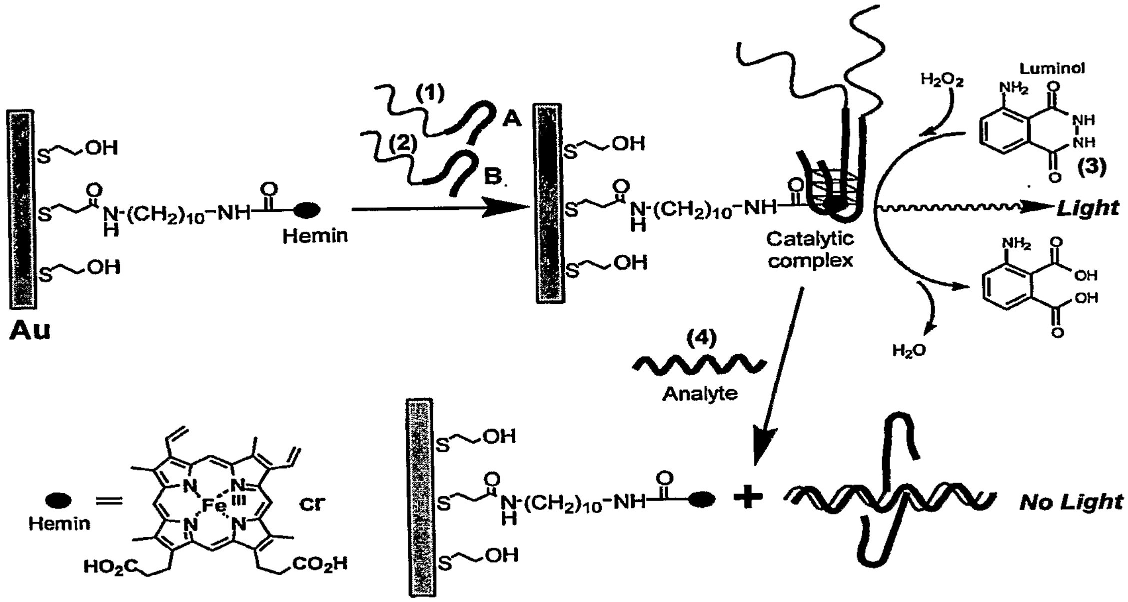
Figure 3



(1) = 5'-CGATTCGGTACTGGCTCAAAATGTGGAGGGT-3'
(2) = 5'-AGGGACGGGAAGAAAGATAATGCGCATGCTCAA-3'
(4) = 5'-TTGAGCATGCGCATTATCTGAGCCAGTACCGAATCG-3'

Scheme 1
Figure 4

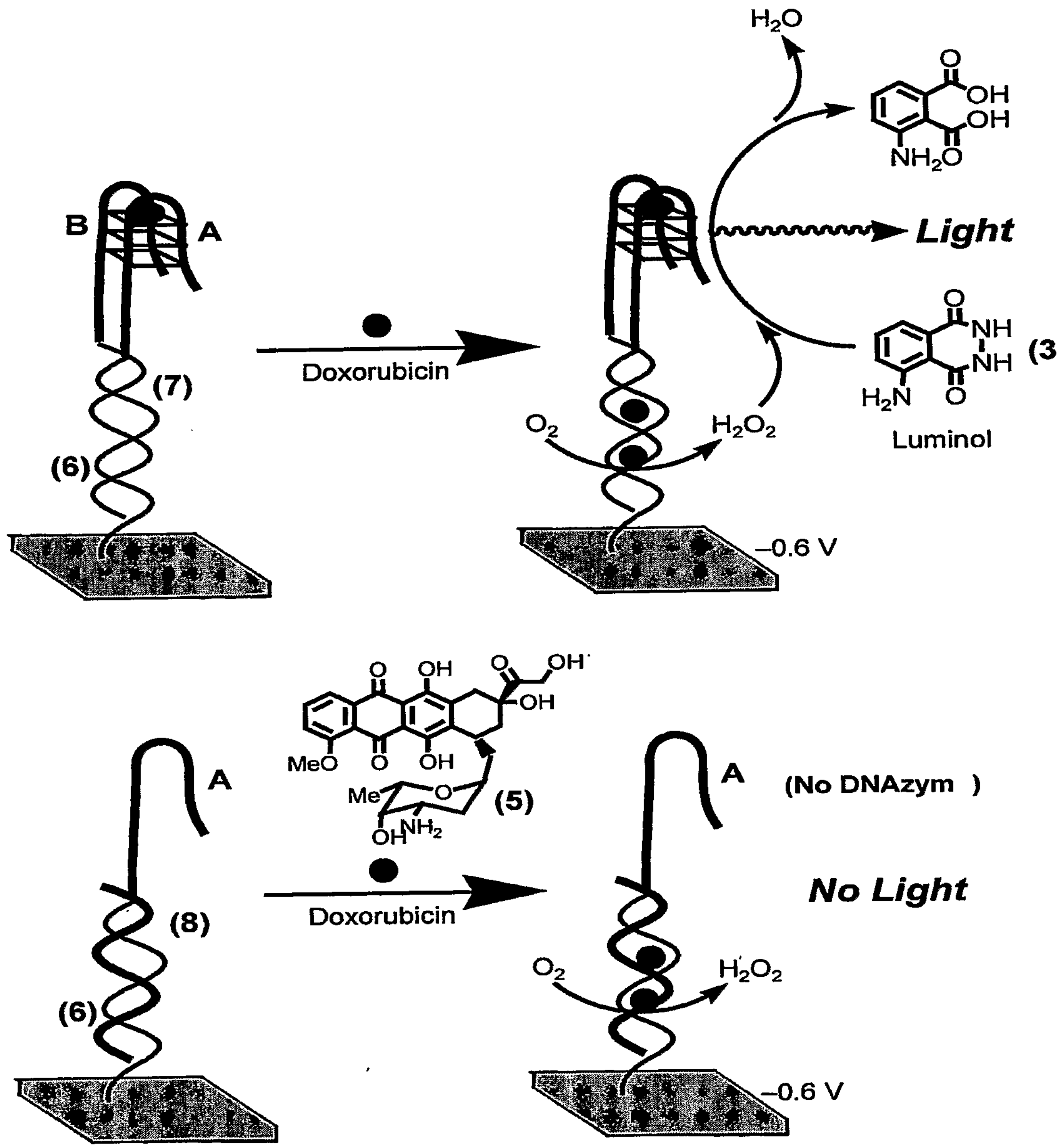
01512474\13-01



(1) = 5'-CGATTCGGTACTGGCTCAAAATGTGGAGGGT-3'
(2) = 5'-AGGGACGGGAAGAAAGATAATGCGCATGCTCAA-3'
(4) = 5'-TTGAGCATGCGCATTATCTGAGCCAGTACCGAATCG-3'

Scheme 2

Figure 5



(6) = 5'-HS$(CH_2)_6$CGATTCGGTACTGGCTCAAAATGTGGAGGGT-3'
(7) = 5'-AGGGACGGGAAGATGAGCCAGTACCGAATCG-3'
(8) = 5'-TGAGCCAGTACCGAATCG-3'

Figure 6

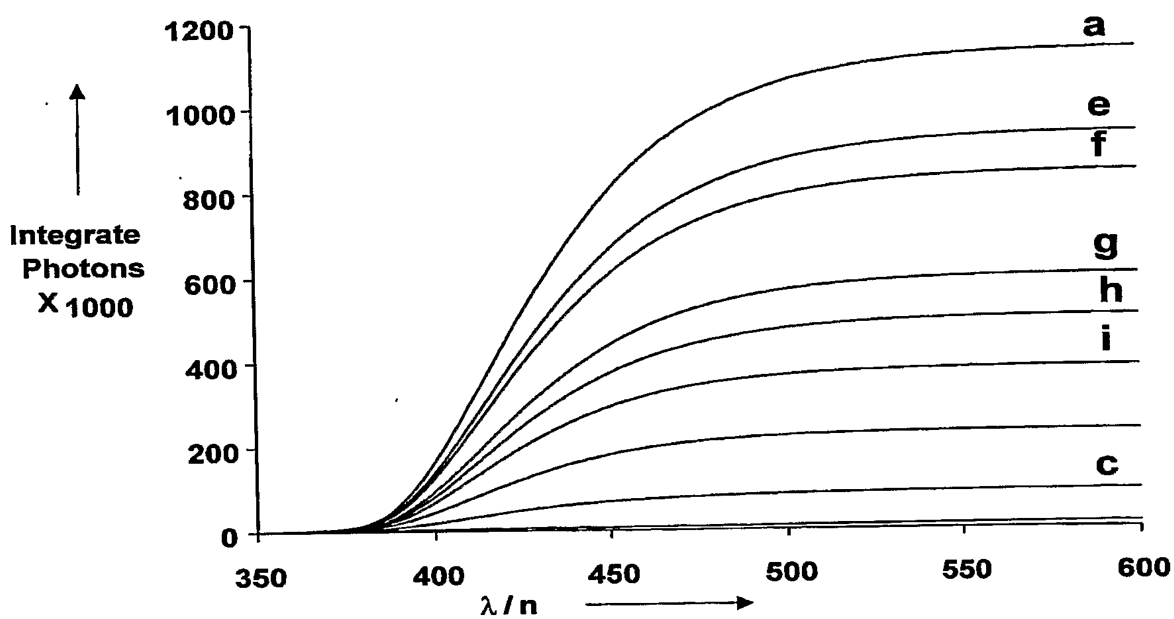

1200
1000
800
600
400
200
0
Integrate Photons X 1000
350
400
450
500
550
600
λ / n
a
e
f
g
h
i
c

Figure 7

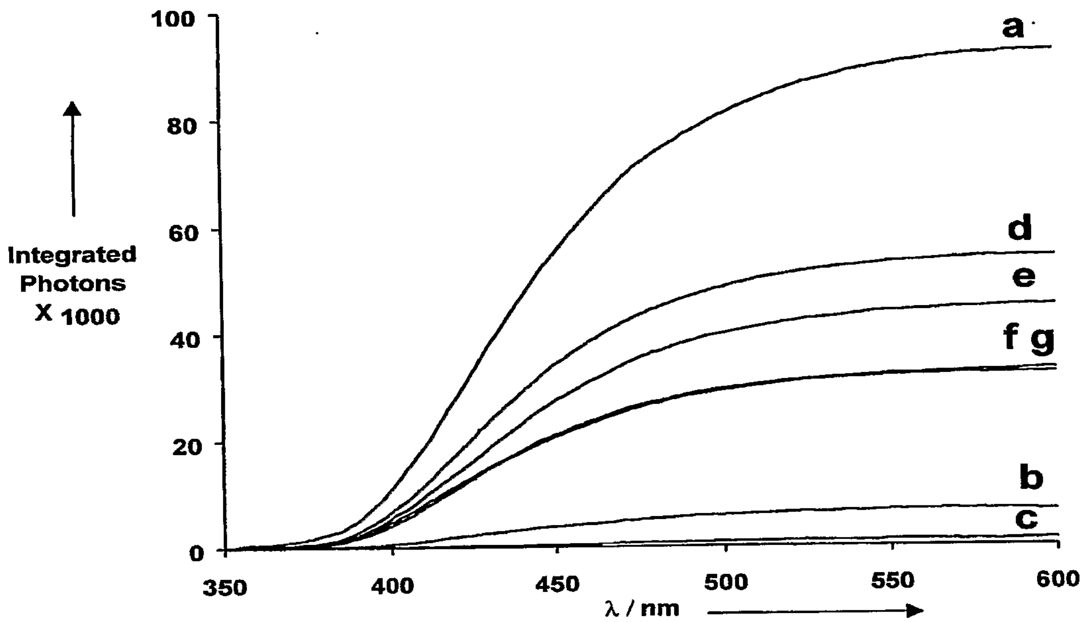


Figure 7

01512474\13-01

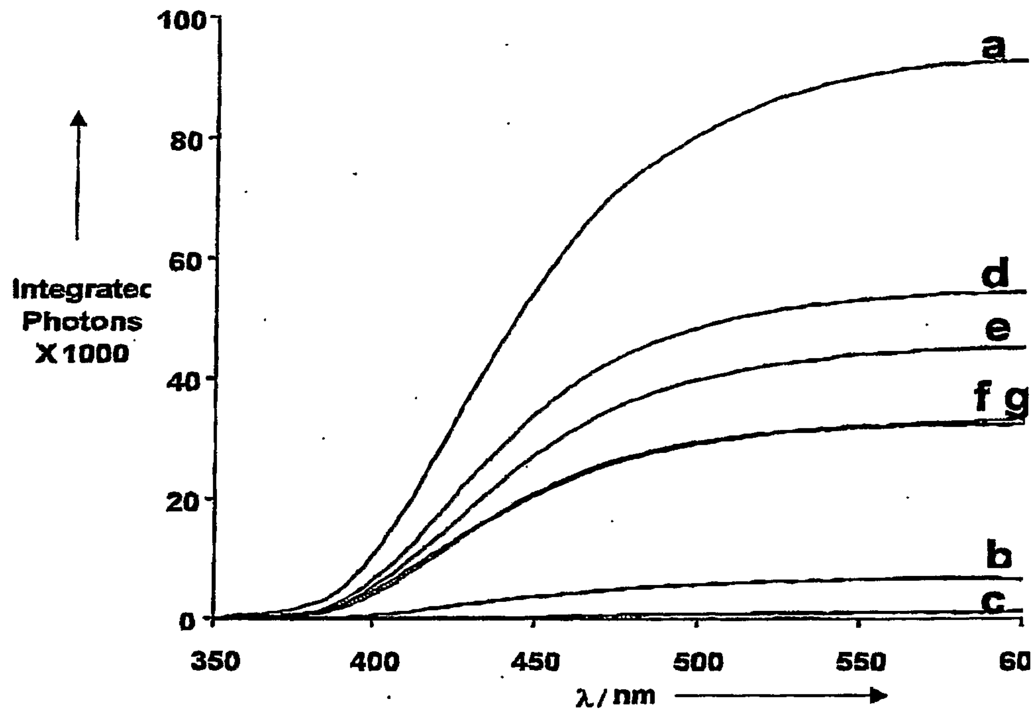

100
80
60
40
20
0
Integratec
Photons
X1000
350
400
450
500
550
60
λ / nm
a
d
e
f g
b
c

Figure 8

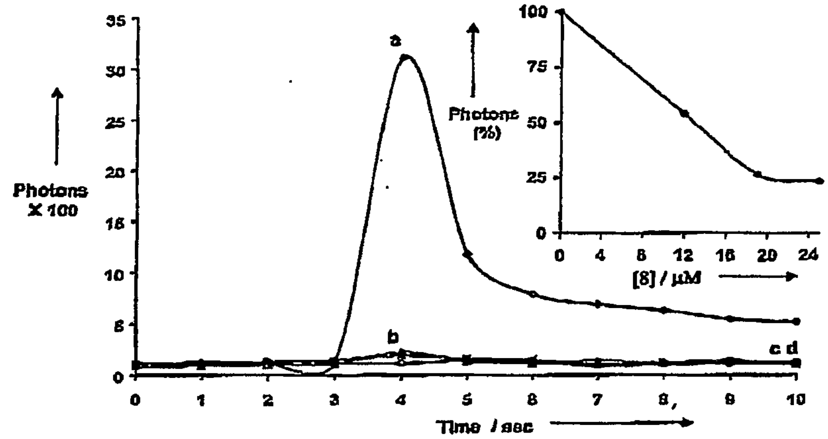
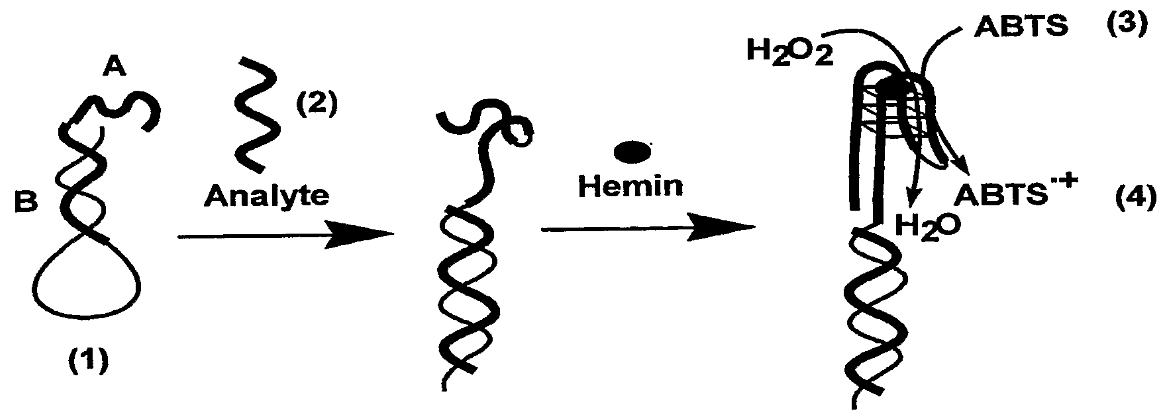


Figure 9

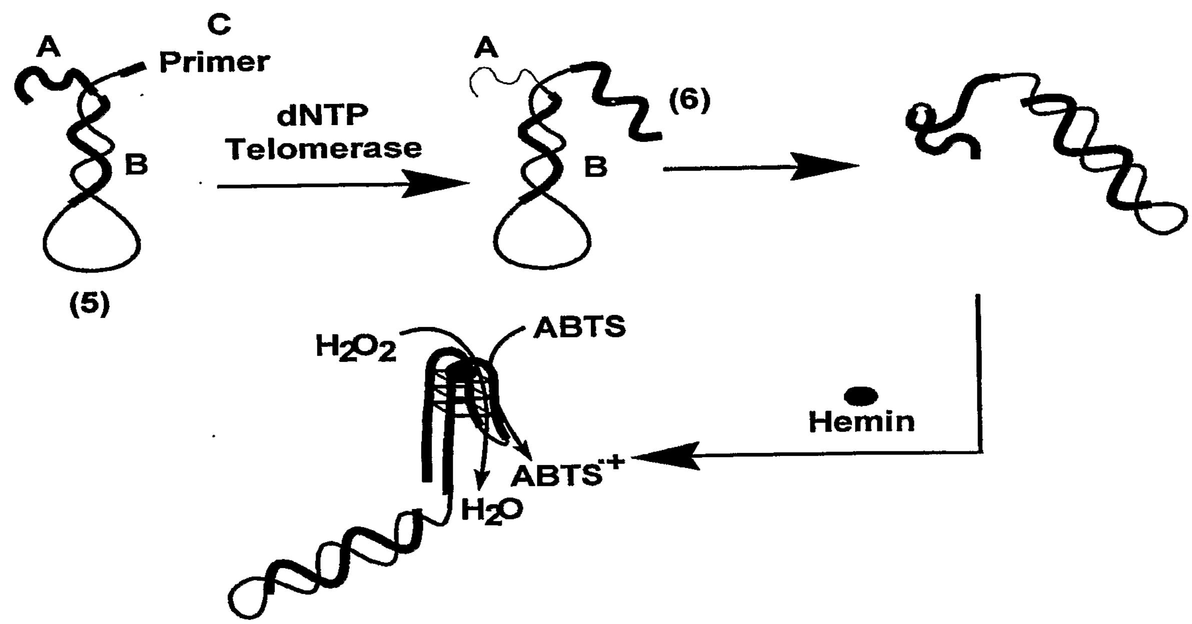
01512474\13-01

(A)



(1)=5'-CCCTACCCAGCCTTAACTGTAGTACTGGTGAAATTGCTGCC
ATTTGGGTAGGGCGGGTTGGG-3'
(2) = 5'-AATGGCAGCAATTTCACCAGTACTACAGTTAAGGC-3'
(2a) = 5'-AATCGCAGCAATTTCACCAGTACTACAGTTAAGGC-3'
(2b) = 5'-AATGGCAGCAATTTCAC GAGTACTACAGTTAAGGC-3'

(B)



(5)=5'-TGGGTAGGGCGGGTTGGGAAATAACCCTAACCCTAACCCT
AACCCTAACCCTAACCCAACCCAATCCGTCGAGCAGAGTT -3'
(6) = 5'-AATCCGTCGAGCAGAGTTAG(GGTTAG)n-3'

Figure 10
01512474\13-01

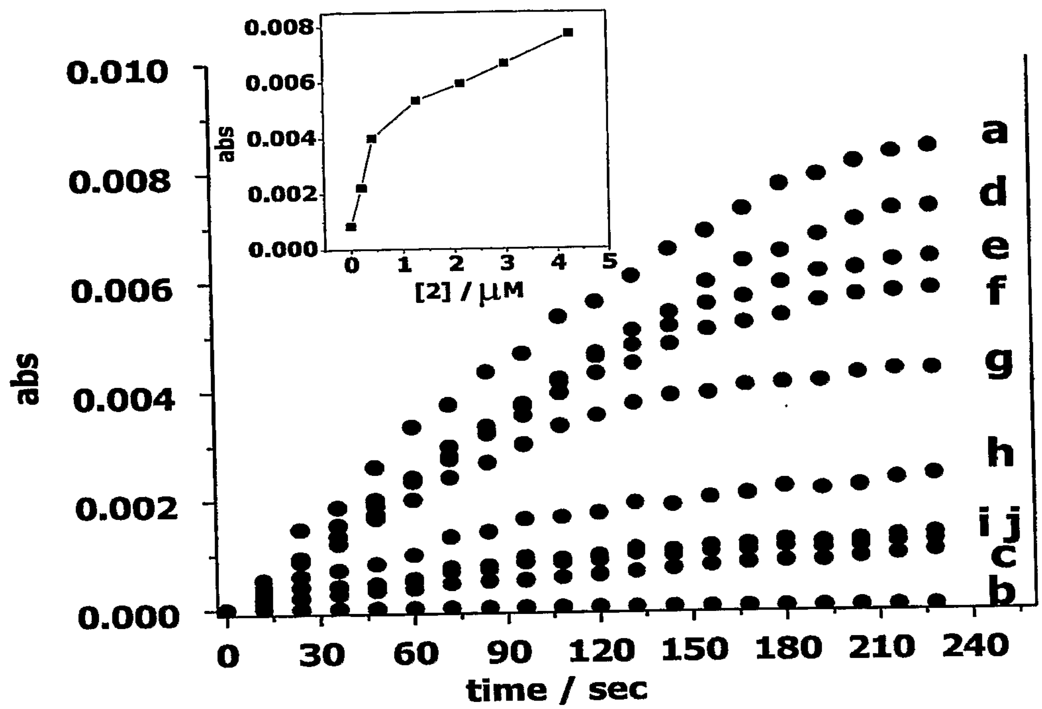
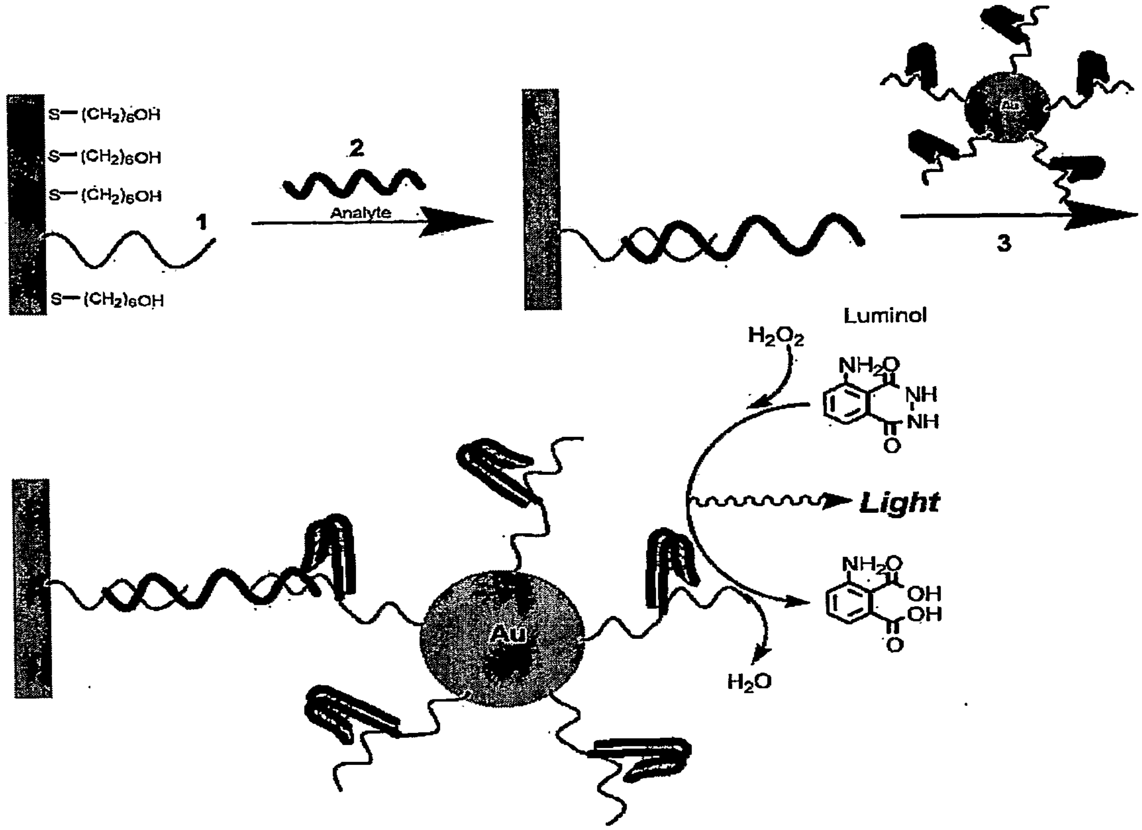

0.010
0.008
0.006
0.004
0.002
0.000
abs
0
30
60
90
120
150
180
210
240
time / sec
a
d
e
f
g
h
i j
c
b
0.008
0.006
0.004
0.002
0.000
abs
0
1
2
3
4
5
[2] / μM

Figure 11



(1) = 5'-HS$(CH_2)_6$CGATTCGGTACTGG-3'
(2) = 5'-TTGAGCATGCGCATTATCTGAGCCAGTACCGAATCG-3'
(3) = 5'-ATGCGCATGCTCAATTTGGGTAGGGCGGGTTGGGTTTTTTTTTTTTTTTTT-3'

Fig 12

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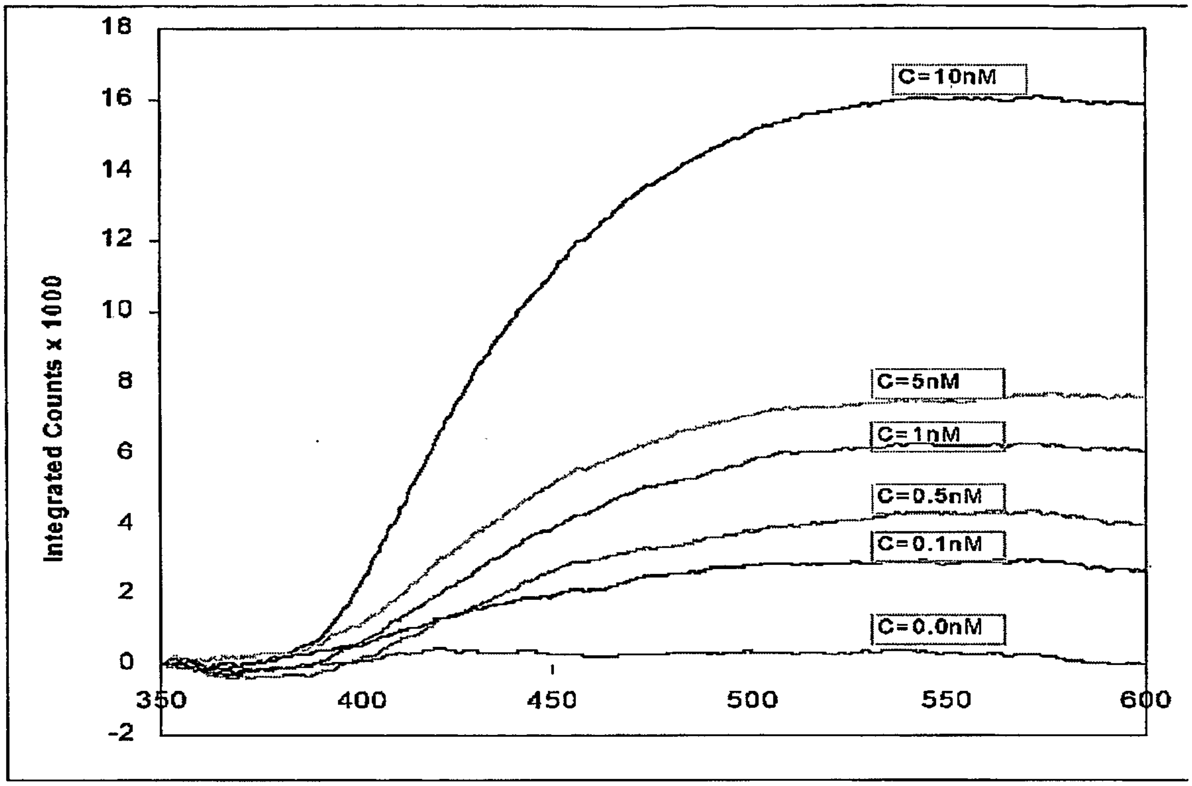
C=10nM
C=5nM
C=1nM
C=0.5nM
C=0.1nM
C=0.0nM
Integrated Counts x 1000
18
16
14
12
10
8
6
4
2
0
-2
350
400
450
500
550
600

Fig 13